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(54) Title: HOMOGENOUS LUMINESCENCE ENERGY TRANSFER ASSAYS

(57) Abstract

The invention relates to improvements of energy–transfer based homogeneous assays, which use time–resolved fluorometry in detection. The specific improvements relate to the type of lanthanide chelate labels used as energy donors, optimized energy acceptors for defined assays, the way energy transfer is measured using optimized filters and time windows, ways to correct all possible interferences derived from samples, use the assay for multi–component analysis and development of simplified assay protocols.

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HOMOGENOUS LUMINESCENCE ENERGY TRANSFER ASSAYS

FIELD OF THE INVENTION

The present invention relates to improvements of energy-
transfer based homogeneous assays, which use time-resolved
fluorometry in detection. The specific improvements relate
5 to the type of lanthanide chelate labels used as energy
donors, optimized energy acceptors for defined assays, the
way energy transfer is measured using optimized filters and
time windows, ways to correct all possible interferences
derived from samples, use the assay for multi-component
10 analysis and development of simplified assay protocols.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to
illuminate the background of the invention, and in
particular, cases to provide additional details respecting
15 the practice, are incorporated by reference.

A great variety of assays based on bioaffinity or
enzymatically catalysed reactions have been developed to
analyze biologically important compounds from various
biological samples (such as serum, blood, plasma, saliva,
20 urine, feces, seminal plasma, sweat, liquor, amniotic
fluid, tissue homogenate, ascites, etc.), samples in
environmental studies (waste water, soil samples)
industrial processes (process solutions, products) and
compound libraries (screening libraries which may comprise
25 organic compounds, inorganic compounds, natural products,
extracts of biological sources, biological proteins,
peptides, or nucleotides, and so on). Some of these assays
rely on specific bioaffinity recognition reactions, where
generally natural biological binding components are used to
30 form the specific binding assay (with biological binding
components such as antibodies, natural hormone binding

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proteins, lectins, enzymes, receptors, DNA, RNA or PNA) or
artificially produced binding compounds like genetically or
chemically engineered antibodies, molded plastic imprint
(molecular imprinting) and so on. Such assays generally
5 rely on a label to quantitate the formed complexes after
recognition and binding reaction and suitable separation
(separations like precipitation and centrifugation,
filtration, affinity collection to e.g. plastic surfaces
such as coated assay tubes, slides of microbeads, solvent
10 extraction, gel filtration or other chromatographic
systems, and so on). The quantitation of the label in free
or bound fraction enables the calculation of the analyte in
the sample directly or indirectly, generally through use of
a set of standards to which unknown samples are compared.

15 The separation and washing needed in most of these assays
make them labor intensive, slow and difficult to automate.
Furthermore the end point measurement does not allow
gathering of kinetic information (associations/
dissociation rates). In cases of low affinity bindings, the
20 affinity may be so low that no physical separation can be
applied without destroying the binding (low affinity
receptors). Particularly in areas, like screenings (high
throughput screening) there is a constant demand for
simpler assays, simplified protocols, which would make
25 automation easier and increase the throughput. That can be
accomplished with homogeneous or non-separations assays.
Homogeneous biomedical assays are defined as assays taking
place in one homogeneous phase. It means that no separate
phases (such as solid phase catching reagents), and no
30 separation is used prior to measurement. That requires a
signal production system that responds to the binding assay
in a way making its direct monitoring possible. Systems
know prior art are e.g. fluorescence polarization assays
applied for small molecular compounds, enzyme-monitored
35 immunoassays (marketed by Syva), various fluorescence
quenching or enhancing assays (for an review see e.g.
Hemmilä, Applications of Fluorescence in Immunoassays,

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Wiley, NY, 1991). Another category of simplified assay
technologies are non-separation assays, which, similarly to
homogenous assays, avoid separation and washing steps. A
good example of such a technology is the scintillation

5 proximity principle marketed by Amersham, which is based on
short distance penetration of radiation particles in assay
medium and solid scintillator coated with catching reagents
(Udenfriend et al, (1985) Proc Natl Acad Sci, 82, 8672 and
Anal Biochem, (1987) 161, 494).

10 Regardless of a great number of homogeneous assay designs
published to day, there are no assays, where the
versatility and sensitivity would match those of a good
separation assay. The reason to that is manyfold relating
to e.g. the different way homogeneous, versus

15 heterogeneous, assay has to be optimized, the control of
low affinity non-specific bindings, and the limitations of
applicability of most of the existing homogenous assay
techniques. In addition, the conventional homogeneous
fluorometric assays are very vulnerable to background

20 interferences derived from various components in the
samples. Fluorescence polarizations assays are interfered
by low affinity nonspecific bindings (e.g. probe binding to
albumin) and autofluorescence of samples.

25 Time-resolved fluorometry (time resolution in time-domain
at micro- or millisecond range) is a perfect measuring
regime for homogeneous assays, because it can totally
discriminate the background fluorescence derived from
organic compounds when long enough delay times (time

30 between pulsed excitation and starting of emission
recording) are used (for a review see. e.g. Hemmilä (1991);
Gudgin Dickinson et al, (1995) J Photochem Photobiol 27,
3). In addition to separation based assays, also a number
of homogeneous time resolved fluorometric assays have been

35 described and patented (Mathis (1995) Clin Chem, 41, 1391;
Selvin et al. (1994) Proc Natl Acad Sci, USA, 91, 10024)

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with their limitation and drawbacks. The aim of the present
invention is i.a. to provide an improvement in time-
resolved homogeneous assay principle based on specific
energy transfer between a long life-time chelate-based
5   donor probe and short life-time (or non-emitting) acceptor
molecule.

Resonance Energy Transfer

The Förster type of nonradiative dipole-dipole energy
transfer (Förster (1948) Ann Physik, 6, 55) takes place
10   between two molecules in condition where their energies
(emission of donor with absorption of acceptor) overlap and
they are at a distance less than 20 nm. The energy transfer
requires a proper orientation of the oscillations of the
molecules. The energy transfer efficiency (Φ_{ET}) is given in
15   the equation:

$$\Phi_{ET} = 1/[1 + (r/R_0)^6]$$

where r is the distance between the donor and acceptor
molecules and R_0 is a distance parameter characteristic of
the donor-acceptor pair and the medium between them.

20   Fluorescence resonance energy transfer (FRET) has been
applied e.g. as an spectroscopic ruler in structural
studies to measure distances within a macromolecule (Stryer
and Haugland (1967) Proc Natl Acad Sci, USA, 58; 719). In
addition to resonance energy transfer, there are also other
25   energy transfer reactions, like simple radiative (where
acceptor absorbs the light emitted by donor), collisional
energy transfer, exchange (Dexter (1953) J Chem Physics,
21, 836) and exciton migration (in crystals). In addition,
the donor emission can be quenched by numerous ways with a
30   number of unrelated compounds having an deactivating effect
on some of the donor's energy levels.

Ullman was the first to describe application of Förster-
type non-radiative energy transfer in bioanalytical assays
based on antibody recognition reaction (Ullman, Scharzberg
and Rubenstein (1967) J Biol Chem, 251; 4172) marketed as
5 FETIA (fluorescence energy transfer immunoassay) by Syva
Co. (US 3,996,345). Development of suitable energy donor-
acceptor pairs are well described in their various reviews
(Ullman and Khanna (1981) Methods Enzymol, 74; 28; Khanna
and Ullman (1980) Anal Biochem, 108, 156). FETIA primarily
10 applies xanthene dyes and derivatives of fluorescein as
donor and rhodamines as acceptors. A great number of
alternative probe pairs have since been developed and
applied in immunoassays (for a review see Hemmilä 1991,
chapter 8.3.4) including practically non-fluorescent
15 derivatives of fluorescein, long life-time delayed
fluorescence emittive eosin (Thakrar and Miller (1982) Anal
Proc, 19, 329), long lifetime fluorescent pyrene (Morrison
(1988) Anal Biochem, 174, 101) and non-emittive charcoal.
FRET has since got wide applications in basic research and
20 in DNA hybridizations (see e.g. Morrison et al (1989) Anal
Biochem, 183, 231; Parkhurst et al (1995) Biochemistry, 34,
285) and other assays were association, dissociation or
distances are to be measured.

The use of temporal discrimination (time-resolution) to
25 avoid the effect of direct excitation of acceptor molecule
was described by Morrison (Morrison (1988) Anal Biochem,
174, 101) with organic donor-acceptor pairs of different
decay times using pyrene as the long excited state donor,
pulsed laser for excitation and phycoerythrin as the short
30 decay-time acceptor (US 4,822,733). In addition to
immunoassays, the time-resolved energy transfer principle
is applied for homogeneous solution hybridization using
fluorescein (Morrison et al (1989) IIIrd International
Symposium on Quantitative Luminescence Spectrometry in
35 Biomedical Sciences, Ghent Belgium) as acceptor. The long
excited state provides the advantage, that specific energy

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transfer can be followed using a delay time, during which
the emission of directly excited acceptor is decayed off.
The combination of different decay times exploited in time-
resolved fluorometry will provide a clear advantage over
5 FRET technologies employing conventional short decay
probes.

Luminescent (fluorescent) lanthanides have long been used
as tools for DNA and protein structural studies (See e.g.
Brittain (1985) Bioinorganic Luminescence Spectroscopy,
10 Schulman (ed), Molecular Luminescence Spectroscopy, Wiley,
NY; Bünzli and Choppin (1989) Lanthanide Probes in Life,
Chemical and Earth Sciences, Theory and Practice, Elsevier
Science Publisher, Amsterdam), applied e.g. for RNA,
ribosomes, DNA and chromatin, termolysin and transferrin,
15 calmodulin, ATPase, nerve membranes, erythrocyte membrane
proteins, phospholipids and liposomes and their fusions
(for an review see Hemmilä 1991). The energy transfer
between lanthanides attached to different sites of a
calcium binding protein, or DNA, have been used as
20 spectroscopic ruler to measure the locations of ion-binding
sites, e.g. with calmodulin using energy transfer between
Eu and Nd (Horrocks and Tingey (1988) Biochemistry, 27,
413). Similarly FRET has been used in chelate studies by
measuring energy transfer from Tb to Eu (Brittain (1978)
25 Inorg Chem, 17, 2762).

Lanthanides and their chelates are good candidates for
time-resolved FRET experiments. Firstly, they have
exceptionally long excited state lifetime (Weissman (1942)
Chem Phys, 10, 214; Whan and Crosby (1962) Mol Spectrosc,
30 8, 315). The energy transfer takes place only between
transitions that are electric dipole. The major transition
in the highly fluorescent Eu chelates, (transition $^5D_0 - {}^7F_2$)
at 612-620 nm is electric dipole "forced" (Bünzli (1989)
Lanthanide Probes in Life, Chemical and Earth Sciences.
35 Theory and Practice, Bünzli and Choppin (ed) Elsevier

Science, Publisher, Amsterdam) and can donate energy. The
transition $^5D_0$ - $^7F_1$ producing emission at 590-595 nm is,
however, magnetic dipole and can not transfer energy
(Dexter, J Chem Phys 21; 836, 1953). In addition,

5   lanthanide emission has isotropic dipolic moment, and hence
the orientation factor becomes less ambiguous (Ando et al
(1992) Biochim Biophys Acta, 1102, 186).

Fluorescent lanthanide chelates have been used as energy
donors already since 1978 by Stryer, Thomas and Meares. For

10 example a Tb dipicolinate chelate reported to have the
critical distance (R_o) of 6.57 nm for rhodamine, 4.46 nm for
eosin and 4.46 nm for NBD (Thomas et al (1978) Proc Natl
Acad Sci, 75; 5746). Typically in an efficient energy
transfer, the overall decay shortens, as shown in the above

15 system, from 2.22 ms to 0.12 ms. Meares et al (1981, 1992)
measured enzyme bound rifamycin using Tb-phenyl-EDTA as
energy donor (Biochemistry 20; 610; Biochemistry 22; 6247).
In addition to fluorescent compounds, also nonluminescent
acceptors have been reported, such as Co (III) and Co (II)

20 (Cronce and Horrocks (1992) Biochemistry 31, 7963). Eu and
Tb chelates have also been tested as energy acceptors, Eu
for coumarin derivative and Tb for salicylate derivative
(Clark et al (1993) Anal Biochem 210, 1). The great number
of available non-luminescent energy acceptors (quenchers)

25 is an additional advantage for lanthanide chelates. They
can be quenched by metallic ions, nitrite (Tanaka et al
(1993) J Photochem Photobiol A: Chem, 74, 15), other
paramagnetic metallic ions and their chelates, free
radicals (Matko et al (1992) Biochemistry 31, 703) and so

30 on.

The use of lanthanides as fluorescent labels in various
forms of bioaffinity assays are well documented in a great
number of reviews and patents (see for example Hemmilä

35 1991, Gudgin Dickson 1995). Examples of the early ideas
about a 'workable' chelate label can be found e.g. in

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following patents (US 4,374,120; 4,432,907; 4,965,211;
4,341,957; 4,058,732 and 4,283,382). A heterogeneous assay
system based on non-luminescent labelling chelates (US
4,808,541) and a dissociative fluorescence enhancement (US
5   4,565,790) has got the widest applications. That system, as
well as system based on fluorogenic ligand and after-assay
cationic saturation (EP-A 354847 and US 4,772,563), does
not suit for homogeneous assays. There are, however, a
great number of stable fluorescent chelates, described in
10   patents and articles, which could be used in time-resolved
FRET assays, such as mentioned in following US patents: .
4,761,481; 5,032,677; 5,055,578; 5,106,957; 5,116,989;
4,761,481 4,801,722; 4,794,191; 4,637,988; 4,670,572;
4,837,169 and 4,859,777. The preferred chelate is composed
15   of nona-dentate chelating ligand, such as terpyridine (EP-A
403593; US 5,324,825; US 5,202,423, US 5,316,909),
terpyridine analogue with one or two five-membered rings
(e.g. pyrazole, thiazole, triazine) (US Appl 08/548,174 and
PCT/FI91/00373). Very well suited chelates are also
20   mentioned in following articles (Takalo et al (1994)
Bioconjugate Chem, 5, 278; Mukkala et al (1993) Helv Chim
Acta, 76, 1361; Remuinnan et al (1993) J Chem Soc Perkin
Trans, 2, 1099; Mukkala et al (1996) Helv Chim Acta, 79,
295; Takalo et al (1996) Helv Chim Acta, 79). Examples of
25   other long decay time labels suitable for the present
invention are the phosphorescent complexes of porphyrin
derivatives with Pd and Pt
(WO 94/10568).

Use of long decay-time lanthanide chelates in various types
30   of homogeneous binding assays are also described, based
e.g. on mixed-ligand chelate (US 4,587,223),
environmentally sensitive chelate (EP 324323) and a chelate
as enzyme substrate (EP 374221).

Since the development of the fluorescence resonance energy
35   transfer based homogeneous assays in 1976, they have

obtained wide applications in diagnostics, commercialized
by Syva corporation (US 3,996,345). Use of lanthanide
chelates as specific labels in time-resolving fluorometric
systems was first described in 1970's by Leif (Leif et al

5   (1976) Automation of Uterine Cancer Cytology 1, GL Weid, GF
Bahar and PH Bartels (ed) Tutorial of Cytology, Chicago IL)
and Wieder (Wieder (1978) Proc. 6th Int. Conf. On
Immunofluorescence and related staining techniques, W
Knapp, K Holubar and G Wick (eds), North-Holland,

10   Amsterdam; US 4,058,732, DE 2,628,158) followed by the
development of working diagnostic systems in 1980's (US
4,565,790). On the other hand, fluorescent lanthanides were
used for fluorescence resonance energy transfer experiments
already in 1978 by Stryer et al (Thomas et al (1978) Proc

15   Natl Acad Sci, 75, 5746; Stryer et al, Annual Review of
Biophysics and Bioengineering; Mullins LJ (ed) Annual
Reviews, Inc; Palo Alto, CA, 1982, vol 11,p 203).
Accordingly, their use as long excited state probes for
FRET immunoassays is obvious, but still an object of

20   patent applications. Wieder and Hale have obtained a patent
on homogeneous time-resolved immunoassays based on various
techniques, including energy transfer and quenchings (EP-A
272320 and WO 87/07955) and Stavrianopoulos (EP-A 242527,
1987) in an assay based on the use of Clq and FRET. Hoffman

25   La Roche has patented a specific application using
lumazine-type of chromophore as donor and a ruthenium
chelate as acceptor for interactions between nucleic acids
(EP 439036) and Cis Bio International applied for an assays
based on Eu or Tb cryptates as donors for phycobiliproteins

30   (WO 92/01225).

The principle of a time-resolved homogeneous assay based on
a specific energy transfer between a long-life time donor
and a short life-time emitting acceptor molecule can be
summarized as follows: The emission duration of a short

35   excited state acceptor, excited during the excited life-
time of a long-decay time donor through the mechanism of
energy transfer, is longer than the decay time of the

directly excited acceptor. This fact permits the use of
temporal discrimination (time-resolution) to avoid the
luminescence from directly excited acceptor. In a
homogenous assay based on energy transfer the long-lived

5   donor is excited with a light pulse (A) (Figure 1). The
luminescence emission of the energy-transfer excited
acceptor is measured (D) after a suitable delay (C), to
avoid interference derived from directly excited acceptor
(B). The long-lived emission from the donor (DE) is avoided

10   by measuring the emission of the energy-transfer excited
acceptor (AE) at a wavelength where the donor emission is
absent or negligible (Figure 2). Hence, in homogenous
bioaffinity assay (receptor-ligand binding, hybridisation
reaction, immunobinding, enzyme substrate binding etc.) the

15   association or dissociation of donor-acceptor pairs can be
followed by measuring the increase or decrease,
respectively, in the signal from the energy-transfer
excited acceptor.

OBJECTS AND SUMMARY OF THE INVENTION

20   An object of the present invention is to provide a
luminescence energy transfer assay comprising a first group
labelled with an energy donor and a second group labelled
with an energy acceptor, wherein the acceptor is either a
short excited state lifetime luminescent label or a non-

25   luminescent label. The increase or decrease, respectively,
in energy transfer from the donor label to the acceptor
label resulting from shortening or lengthening,
respectively, of the distance between said labels, is
measured. According to the invention, the donor is a

30   luminescent lanthanide chelate.

According to one aspect of the invention the assay is based
on a reaction resulting in lengthening of the distance
between the labelled donor and acceptor groups. According
to this invention, the donor luminescent lanthanide chelate

35   has a long excited state lifetime.

According to another aspect of the invention the assay is based on a reaction resulting in either shortening or lengthening of the distance between the labelled donor and acceptor groups and the donor is a luminescent lanthanide

5   chelate having a long excited state lifetime. According to this aspect of the invention the long excited state lifetime luminescent lanthanide chelate has one or more of the following properties:

- a high luminescence yield,

10   - an excited state lifetime of 1 ms or more, and
- an emission distribution optimized for energy transfer.

According to yet another aspect of the invention, two or more analytes are simultaneously measured, wherein each

15   analyte is measured by a specific donor-acceptor pair.

According to yet another aspect of the invention, the luminescent or non-luminescent label of the acceptor is attached to a solid carrier providing a large surface to which said second group is bound or can be bound via

20   suitable covalent or non-covalent means.

According to yet another aspect of the invention, the acceptor is lipophilic compound anchored to a cell membrane or cell membrane fragment or the acceptor is made lipophilic by conjugating with a long chain hydrocarbon

25   such as a fatty acid.

According to yet another aspect of the invention, the instrument used to measure energy transfer is able to follow multiple parameters simultaneously or consecutely, such as absorbances at the wavelenghts of excitation or

30   emissions, luminescence of different durations, scattering and luminescence decay times, in order to monitor and correct any interferences the sample matrix may have to excitation, energy transfer or emissions.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the luminescence versus time after donor
excitation with a light pulse (A) of directly excited
acceptor (B) and the luminescence emission of the energy-
5 transfer excited acceptor (D) after a suitable delay (C).

Figure 2 shows the luminescence intensity versus emission
wavelength of donor (DE) and acceptor (AE).

Figure 3 shows a standard curve for the homogenous energy
transfer assay of ßhCG (ß-subunit of human chorionic
10 gonadotropin).

Figure 4 shows a standard curve for the homogenous energy
transfer assay of PSA (prostate specific antigen).

Figure 5 shows a standard curve for the homogenous energy
transfer assay of ßhCG using indirect labelling.

15 Figure 6 shows a standard curve for the homogenous energy
transfer assay of ßhCG using indirect labelling and an
acceptor different from that of Figure 5.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to improvements of energy-
20 transfer based homogeneous assays, which use time-resolved
fluorometry in detection. The specific improvements relate
to the type of lanthanide chelate labels used as energy
donors, optimized energy acceptors for defined assays, the
way energy transfer is measured using optimized filters and
25 time windows, ways to correct all possible interferences
derived from samples, use the assay for multi-component
analysis and development of simplified assay protocols.

The terms "first group" and "second group" shall be
understood to include any component such as bioaffinity

recognition component (in reactions where the distance
between the groups decreases, e.g. in bioaffinity
reactions) or a part of a molecule or substrate (e.g. the
distal ends of a peptide molecule the cleavage of which
5 will separate the two labelled groups from each other).

The term "luminescence" shall cover fluorescense,
phosphorescence, chemiluminescence, bioluminescence and
electro-generated luminescence, photoluminescence,
radioluminescence, sonoluminescence, thermoluminescence and
10 triboluminescence.

A preferred arrangement in assays, where association is to
be measured is to use luminescent, short decay time
acceptor and long decay time lanthanide chelate based donor
and follow the emission of acceptor molecule using a delay
15 time in the time-resolved fluorometry to avoid the
interference of acceptors direct luminescence (emanating
from direct excitation of acceptor). It is desirable to
construct the assay in a way that acceptor molecules are in
excess (with time-resolved mode, their interference is
20 negligible) and the association of binding reagents creates
an increase in signal.

For such a system the preferred chelate label has to have
high luminescence yield ($\epsilon x\Phi$ >2000), long excited state
lifetime (preferably over 1 ms) and emission distribution
25 optimized for energy transfer. The ligand field around the
chelated ion has to be such that e.g. with Eu chelates over
70 % of emission is at $^5D_0$ - $^7F_2$ (at 610 - 620 nm range) and
not at 590 nm range (compare e.g. emissions of Eu cryptate,
WO 92/01225 and those of bis-iminoacetate derivatives of
30 terpyridines, US 5,324,825; US 5,202,423 and US 5,316,909).
In preferred chelates the useless magnetic dipole
transition at 590 nm and emission around 700 nm are
suppressed (Li and Selvin, J Amer Chem Soc 117; 8132,
1995). Particularly good chelates for the present
35 application are Eu chelates formed with multichromogenic

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polycarboxylates, having high molar absorption coefficient
(ϵ), very long excited state lifetime and good quantum
yield (Φ) (Takalo et al. Helv Chim Acta 79; 789, 1996). In
addition to Eu, Tb is particularly promising energy donor,

5   when its highly luminescent chelates are used. A preferred
Tb chelate is composed of terpyridine derivatives
containing the binding side at the iminodiacetate group
(Mukkala et al, J Alloys Compounds 225; 507, 1995) or
otherwise a binding arm well isolated from the light

10 absorbing aromatic structure. Particularly good chelates
for that applications are terpyridine derivatives where one
or two pyridine rings are replaced with pyrazole (US
08/548,174) or triazole and thiazole rings
(PCT/FI91/00373). In addition to Eu and Tb, the use of Sm

15 would give the possibility to make double- or triple-label
homogeneous energy transfer assays. Sm has the advantage,
that it can donate energy at a rather high wavelength, the
major emission of a highly luminescent chelate being at 643
nm, giving the opportunity to continue with the wavelength

20 scale up to near IR (a good collection of near-IR emitting
fluors have become commercially available from different
sources). A preferred stable chelate of Sm is composed of
multiple forms of 1,3-diketones, such as described by
Savitsky (Savitsky et al, SPIE 2388; 429, 1995). An

25 alternative third choice (third label) is the
phosphorescent Pt or Pd coproporphyrins emitting a long
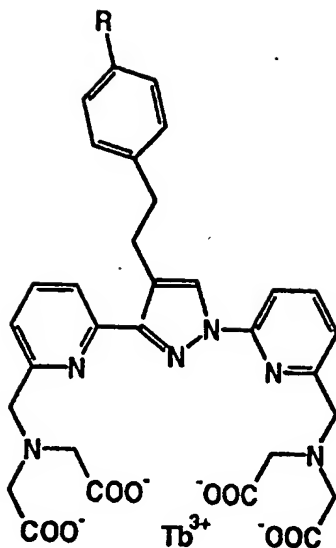lifetime phosphorescence at 650-660 nm
(WO 94/10568).

A particularly preferred group of long excited state
30 lifetime lanthanide chelates are Compounds 1 to 12 the
structure of which is dislosed in Scheme 1 to 5. In the
schemes λ_{exc} means excitation wavelength, τ decay time and ϵ
x Φ luminescence yield. The values given in the schemes are
measured from the compound coupled to a protein.
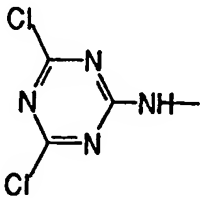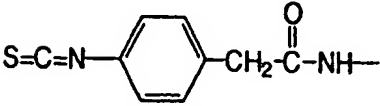
SCHEME 1
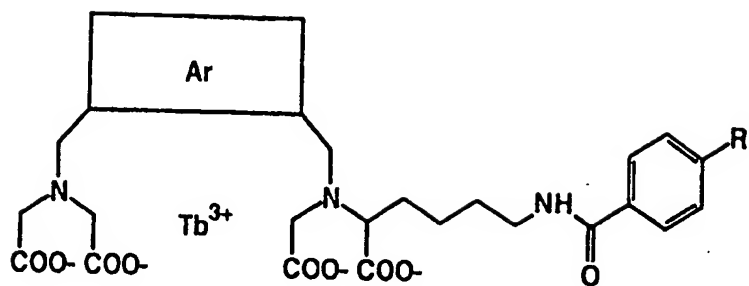
Ar	R	λ_{exc} [nm]	τ [µs]	$\varepsilon \cdot \Phi$
COMPOUND 1	—N=C=S	328	1350	3860
COMPOUND 2	—N=C=S	318	2720	4560
COMPOUND 3	—N=C=S	315	2930	3770
COMPOUND 4	—NH (triazine with Cl, Cl)	310	2660	4050

SCHEME 2

R	λ_{exc} [nm]	τ [μs]	$\varepsilon \cdot \Phi$
COMPOUND 5	333	1260	2060
COMPOUND 6	336	1110	3690

SCHEME 3

R	R'	λ_{exc} [nm]	τ [µs]	$\varepsilon \cdot \Phi$
—N=C=S COMPOUND 7	—OCH₂COO⁻	335	1060	5950
—NH (triazine with 2 Cl) COMPOUND 8	—OCH₂COO⁻	336	1040	5190
—N=C=S COMPOUND 9	(sugar group)	330	1000	3790

SCHEME 4

	λ_{exc} [nm]	τ [μs]	$\varepsilon \cdot \Phi$
COMPOUND 10	319	1050	4370

SCHEME 5

R	λ_{exc} [nm]	τ [μs]	$\varepsilon \cdot \Phi$
—N=C=S COMPOUND 11	340	1400	2100
—NH (triazine with Cl substituents) COMPOUND 12	340	1590	2600

A preferred acceptor molecule for association assay is
highly luminescent (with quantum yield as near unity (1)
as possible) with high molar absorption coefficient
(preferably over 100 000). The absorption of the acceptor
5 has to overlap with the energy-donating emission of donor,
i.e. at 611-620 nm for Eu, 545 nm for Tb, 643 nm for Sm. A
preferred feature of the acceptor is its sharp emission at
a wavelength where donor does not have any emission (such
as 574-575 nm for Tb, 660-670 nm for Eu and around 675 for
10 Sm). The decay-time of the acceptor should be below 1μs. In
addition the acceptor has to be attached to binding
partners, either directly or indirectly (e.g. via anti-
binder antibody, lectin, avidin etc.), covalently or
noncovalently. As covalently conjugated probes, e.g. those
15 of xanthene dyes (rhodamine, tetramethylrhodamine, Texas
Red™ (see e.g. Hemmilä, 1991, Haugland, Handbook of
Fluorescent Probes and Research Chemicals, Molecular Probes
1992-1994, Mayer and Neuenhofer, Angew Chem Int Ed Engl 33;
1044, 1994), some carbocyanines (Cy3.18) (Mujumbar et al,
20 Bioconjugate Chem 4; 105, 1993, Southwick et al, Cytometry
11; 418,1990) and even some porphyrins (Camman,
DE 4,216,696) can be used. B-phycoerythrin, due to its very
high molar absorption coefficient (2,410,00 at 546 nm) and
quantum yield (0.98) makes it very suitable for energy
25 transfer assays, despite its large size (MW 240,000).

A good acceptor for Eu should have absorption maximum at
611-620 nm range. Good acceptor for that wavelength range
can be found particularly from the group of IR emittive
dyes (for reviews see Haugland Molecular Probes, Miller,
30 Spectroscopy Europe 5; 34, 1993, Patonay and Antoine, Anal
Chem 63; 332A, 1991, Southwick et al, Cytometry 11; 418,
1990, Mujumbar et al, Cytometry 10; 11, 1989 and Mujumbar
et al, Bioconjugate Chem 4; 105, 1993, Papkovsky, Appl
Fluorescence Technol III; 16, 1991, Schindele and Renzoni,
35 J Clin Immunoassay 13; 182, 1993). These types of compounds
include phthalocyanines, porphyrins, cyanine dyes (Cy-5,

Cy5,18, Cy5,29) (Selvin et al, J Amer Chen Soc 116; 6029,
1994, Mujumbar et al, Bioconjugate Chem 4; 105, 1993),
conjugated xanthenes (rhodamine 800) (Imasaka et al, Anal
Chem 61; 2285, 1989), squarates (Chang, EP176,252),

5   methylene blue, Nile blue and oxazines (Imasaka et al, Anal
Chem 61; 2285, 1989), indocyanine green (Imasaka et al,
Anal Chem 62; 363A, 1990). Some of the phycobiliproteins
are good candidates also for Eu acceptors, particularly A-
PC, C-PC and R-PC. A preferred energy acceptor for Sm or

10   for coproporphyrins are the IR emittive dyes (see reviews
mentioned above).

A preferred way to ascertain small distances between donor
probe labelled ligand or binding reagent and acceptor probe
labelled binding reagent is to use activated probes coupled

15   directly to binding reagent (e.g. acceptor labelled
receptor protein, antibody or other binding protein). An
alternative way is to use indirect labelling, using e.g.
anti-binder (such as anti-receptor) antibodies labelled
with the acceptor, use of biotinylated binder and acceptor

20   labelled (strept)avidin or to employ other bioaffinity
reactions to bring acceptor molecules in the vicinity of
actual binding site, where the donor-labelled component
either directly or indirectly will be bound.

A preferred way with impure, membrane-bound receptors is to

25   use lipophilic membrane probes and stain all the membranes
near or around the receptor with acceptor molecules.
Suitable probes attached to long aliphatic hydrocarbon
chain, such as e.g. oxacarbocyanine (Molecular Probes) are
suitable for such staining.

30   A further alternative, which would avoid separate labelling
of binding components for each particular assay, is to use
solid carriers (polymers, ceramics or glass or the like)
such as universal catching surfaces containing high
concentration of acceptor molecules. Suitable solid

35   carriers can be e.g. beads or particles with a diameter up

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to 1500 μm or any solid surface. Microbeads labelled with a
wide variety of luminescent probes are available from
different sources. A preferred probe used in the carrier is
a hydrophobic compound, having negligible solubility to
5   water to avoid leakage. A variety of probes suitable for
such labelling can be found amongst scintillator and laser
dyes. With highly luminescent beads the great number of
acceptor molecules may compensate the long distance after
bead coating, and the luminescent bead actually provide a
10   energy accepting surface. When the bead can absorb most of
the donor emitted light, a simple radiative enery transfer
can be applied, in which the energy transfer is a function
of space angle and critical distance with 10 μm beads is in
the range of micrometers. For FRET-based assay, however,
15   when the plastic is first coated with the binding proteins
(e.g. agglutinin) to immobilize membrane receptors, may
result in inefficient energy transfer due to long
distances. A preferred arrangement is thus to use surface
activated beads and use part of the reactive groups for
20   coupling with acceptor molecules, or use acceptor-labelled
binding surface (such as rhodamine labelled agglutinin) or
to label coated protein afterwards with acceptors.

In homogeneous assay of a association reaction
(immunobinding, receptor-ligand binding, hybridizations
25   reaction, enzyme-substrate binding etc.) the preferred way
to measure binding is to follow acceptor signal increase.
The acceptor signal is measured using a filter optimized
for the donor used, having good transmission at the
wavelength of acceptor, but more importantly, absolutely
30   well blocked for each emission lines of the donor. The
filter should not leak any emission emanating form the main
emission line of donor (such as 545 or 490 nm of Tb and
613-615 nm of Eu). In addition the energy transfer filter
has to be situated at wavelength area, where there are no
35   minor emission lines with the used donor. Use of suitable
delay avoid the interference derived from direct excitation
of acceptor (the optimal delay depends on the length of

excitation pulse used, but should be at least ten times
longer).

The decay of the energy transfer excited acceptor is a
function of the decay of donor and the energy transfer
5 efficiency. Thus, during the assay (such as competitive
binding assay or non-competitive assay) the overall decay
is not constant, but is a function of the analyte. In
association assays where specific binding is low and energy
transfer efficiency less than 1 %, the decay time of energy
10 transfer emission of acceptor is quite constant and equal
to the decay time of donor. The delay and counting times
for such measurement is not critical. For assays of higher
efficiencies, the decay time decreases upon binding, and
steeper response can be obtained keeping short delay time
15 and reasonable short counting time. On the other hand, if
donor emission is followed, steeper response is obtained
using long delay time, because when energy transfer occurs,
the total donor emission both decreases and its decay time
shortens. For optimized results in any assay, it is
20 advisable to optimize counting windows according to assay
type, specific binding percentages and energy transfer
efficiencies.

In screening of various compounds or samples, the sample
may have a signal decreasing effect. The sample may, for
25 example, absorb excitation light, emission light or cause
other types of quenchings. In a multi-label counter, such
as Wallac 1420 VICTOR, the interferences can be controlled
by various methods, including (1) decay analysis (2)
simultaneous photometric monitoring of sample absorption at
30 the wavelengths of excitation and emission, (3)
simultaneous measurement of both signals, donor and
acceptor, and calculating their correlation and comparing
to control with same amounts of donor and acceptor without
binding reaction or (4) kinetic measurement during binding
35 following the change of energy transfer or signals. With
binding assays of high efficiency, the analysis of decay

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time is an independent parameter not affected by color
quenching of neither the excitation nor emission. The decay
change can be followed both for donor or for acceptor
(decrease in decay when energy transfer increases). A
5   preferred way to quarantee nondisturbed analysis with all
samples is to use suitable combination of all the above
mentioned correction methods.

According to a preferred embodiment, the energy transfer is
registered by a time-resolved fluorometer using a
10 predeterminated instrumental setting avoiding cross talk or
donor emission to the channel used for acceptor emission
measurement when there is no energy transfer.

Preferably, the instrument automatically corrects any
attenuation of excitation the sample may cause by following
15 simultaneously the absorbances of the samples diluted into
assay mixture and correcting the emission readings
according to excitation or emission attenuation by sample
absorption.

The measured acceptor signal can be normalized by recording
20 also the donor emission which can be compared to zero
calibrator, the lowering of signal without concomitant
increase in acceptor luminescence being recorded and
corrected.

The decay time of either donor or the acceptor can be
25 registered and used as the measuring parameter.

The decay time of the donor or the acceptor can be utilized
in correction of sample interferences.

A second type of assay where improved energy transfer
assays can be applied, are assays where dissociations, and
30 not associations, are followed. Such assays can be e.g.
competitive immunoassays (or other specific binding assay)
where antibody-antigen complex is preformed and the

addition of analyte causes new equilibrium formations
(ligand replacement assays). Another group of such assay
are the assays of cleaving enzymes, such as peptidases
(e.g. HIV peptidases), proteases and other enzymes (e.g.
5 nucleotide hydrolyzing enzymes, helicase, etc.). In
homogeneous measurement of dissociation, the preferred
technology is to follow the increased emission of the
donor.

Preferred chelates for assays based on dissociation are to
10 a great extend same as described above for association
based assays. Terbium, however, is often a better choice,
because it can be quenched with a variety of compounds,
including metal ions, nitrogen compounds such as azide,
nitride (Tanaka et al, J Photochem Photobiol 74; 15, 1993)
15 or radicals (spin labels, such as Doxyl, Proxyl or Tempo
and other N-O compounds containing unpaired electron)
(Matko et al, Biochemistry 31; 703, 1992). Good Tb chelates
suitable for the present invention are mentioned above.

A preferred acceptor for dissociation measuring assay is a
20 good energy acceptor causing as efficient donor quenching
as possible. The acceptor can be luminescent, but does not
have to be. A preferred FRET acceptor has good acceptor
properties (R_0 over 5 nm), but does not have to be highly
luminescent. In addition to luminescent compounds mentioned
25 above, the good acceptor can be a respective compound made
non-luminescent with heavy atom conjugations (such as
erythrosin) or suitable other substituents (see. e.g.
Khanna and Ullman, Anal Biochem 108; 156, 1980, Ullman and
Khanna, Methods Enzymol 74; 28, 1981). To avoid steric
30 interference (e.g. with HIV peptides labelled both with
donor and with acceptor), the acceptor has to be small, and
small molecular quenchers, such as spin labels, are
preferred. Use of larger acceptors possibly requires
indirect approach, i.e. use of small affinity label (small
35 hapten, biotin) and separate step for quenching (addition
of acceptor labelled anti-hapten of avidin), which may have

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to be done after the actual dissociation reaction (end
point detection). A preferred assay design for e.g. a
peptidase is to use peptide substrate labelled at one end
with a fluorescent terbium chelate and at the other end
5   with free radical. The choice between spin quenching and
FRET dipole-dipole energy transfer depends on the actual
distance between the labels in unhydrolyzed peptide. With
small distances (below 2 nm) free radical (spin) is
preferred. If the distance of the two probes would be more
10 than 5 nm, a dipole-dipole energy transfer is preferred.

When designed to be quenched in associated form (such as
quenched substrate), the donor luminescence, and its decay
time is increased upon dissociation. So the preferred delay
time in measurement is long, to avoid detecting of possible
15 remaining luminescence of quenched donor. These assays,
similar to association assays, are sometimes prone to
sample interferences (e.g. color quenchings), and the
problems can be solved similarly as described above.

EXAMPLES

20 The invention is further demonstrated by the following non-
limiting examples.

Example 1

Labelling of anti-ßhCG (human chorionic gonadotropin)
antibody (code F19-9C1) with the fluorescent Tb chelate
25 Compound 6

The energy donor Compound 6 was conjugated to anti-ßhCG
antibody (code F19-9C1, Wallac Oy, Turku, Finland) in the
following way: anti-ßhCG antibody (5mg/ml) was incubated
with a 30-fold molar excess of Compound 6 in 50mM carbonate
30 buffer pH 9.5 over night at +4 °C. The labelled antibodies
were separated from unreacted Compound 6 by gel filtration
(Sepharose 6B with Sephadex G50 overlay, 0.5x70 cm,

Pharmacia, Uppsala, Sweden) with Tris (50 mM Tris-HCl, pH 8
containing 0.9% NaCl) as eluetion buffer. The labelled
antibody was analyzed and found to contain 4.7 molecules of
Compound 6 per antibody. Labelled antibody was kept in Tris
5 buffer, pH 7.4 containing 0.1% bovine serum albumin.

Example 2

Labelling of anti-ßhCG (code M15294) antibody with
tetramethylrhodamine isothiocyanate (TRITC)

The energy acceptor TRITC (Molecular Probes Inc, Eugene,
10 OR) was conjugated to anti-ßhCG antibody (code M15294,
Wallac Oy) in the following way: anti-ßhCG antibody
(5 mg/ml) was incubated with a 15-fold molar excess of
TRITC in 50 mM carbonate buffer pH 9.5 over night at +4 °C.
The labelled antibodies were separated from unreacted TRITC
15 by gel filtration (Sepharose 6B with Sephadex G50 overlay,
0.5x70 cm, Pharmacia) with Tris (50 mM Tris-HCl, pH 8
containing 0.9% NaCl) as eluetion buffer. The labelled
antibody was analyzed and found to contain 1,7
tetramethylrhodamine per antibody. Labelled antibody was
20 kept in Tris buffer, pH 7.4 containing 0.1% bovine serum
albumin.

Example 3

Homogenous energy transfer assay of ßhCG

In this two-site immunoassay, 50 μl of ßhCG standard in the
25 wells of 96-well plates was incubated with 100 ng of
Compound 6 -labelled antibody (donor) and 100 ng of
tetramethylrhodamine labelled antibody (acceptor) in 200 μl
Tris buffer (50 mM Tris-HCl, pH 7.4, containing 0.9 %
NaCl). The two anti-ßhCG antibodies used are directed
30 against different specific antigenic sites on the ßhCG
subunit. The ßhCG standards were from a commercially
available kit for the measurement of free ßhCG in serum

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(Wallac Oy). The reaction mixtures were incubated for 30
min at room temperature on a 96-well plate shaker. The
tetramethylrhodamine fluorescence from the formed complexes
was measured in a time-resolved fluorometer (excitation at
5   340 nm, emission at 572 nm +- 3 nm, delay time 50 µs,
window time 100 µs, cycle time 1 ms), model 1420 VICTOR
(Wallac Oy) A standard curve for the energy transfer assay
of ßhCG is presented in Figure 3.

Example 4

10   Homogenous energy transfer assay of PSA (prostate specific
antigen)

Two antibodies (Wallac Oy) recognising different epitopes
on PSA were labelled with Compound 1 and TRITC,
respectively. Antibody labelling were carried out as
15   described in example 1 and 2. In this two-site immunoassay,
50 µl of PSA standard (from the commercially available PSA
assay kit DELFIA PSA) was incubated together with 100 ng of
Compound 1 (donor) labelled antibody and 100 ng of
tetramethylrhodamine labelled antibody (acceptor) in 200 µl
20   Tris buffer pH 7.4. The assay mixtures were incubated for
30 min at room temperature under continous shaking. The
tetramethylrhodamine fluorescence from the formed
complexes (sandwiches) was measured in the Wallac 1420
VICTOR time-resolved fluorometer (Excitation at 340 nm,
25   emission at 572 nm +- 3 nm, delay time 50 µs, window time
100 µs, cycle time 1 ms). The standard curve for the
homogenous energy transfer assay is documented in Figure 4.

Example 5

Homogenous energy transfer assay of ßhCG with tetramethyl-
30   rhodamine-avidin as acceptor

This two-site immunoassay was performed as described in

Example 3. Instead of using tetramethylrhodamine-labelled
antibody (acceptor), biotinylated antibody and
tetramethylrhodamine-avidin (acceptor) was used. The
tetramethylrhodamine-avidin (Molecular Probes) reacts and
5   forms stable complexes with the biotinylated antibody. The
anti-ßhCG antibody (code M15294) was biotinylated according
to the instructions from the biotin reagent supplier
(Biotinylating Reagent 732494, Boehringer Mannheim,
Mannheim, Germany). The tetramethylrhodamine fluorescence
10   from the formed complexes (sandwiches) was measured in the
Wallac 1420 VICTOR time-resolved fluorometer (excitation at
340 nm, emission at 572 nm +- 3 nm, delay time 50 µs,
window time 100 µs, cycle time 1 ms). The standard curve
for the homogenous energy transfer assay is documented in
15   Figure 5.

Example 6

Homogenous energy transfer assay of ßhCG with
R-phycoerythrin-avidin as acceptor

In this experiment R-phycoerythrin-avidin (Molecular
20   Probes, Eugene, OR, USA) was used instead of
tetramethylrhodamine-avidin, otherwise the experiment was
performed as in Example 5. The R-phycoerythrin fluorescence
from the formed complexes (sandwiches) was measured in the
Wallac 1240 VICTOR time-resolved fluorometer (Excitation at
25   340 nm, emission at 572 nm +-3 nm, delay time 50 µs, window
time 100 µs, cycle time 1 ms). The standard curve for the
homogenous energy transfer assay is documented in Figure 6.

Example 7

Homogenous energy transfer assay of progesterone

30   Monoclonal anti-progesterone antibody (Wallac Oy) was
labelled with Compound 2 according to Example 1.
Progesterone-biotin derivative (Wallac Oy) was used at a

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concentration of $5/K_{aff}$ (K_{aff} = affinity constant of the
progesterone antibody used) and about 2 times more
tetramethylrodamin-avidin (from Molecular Probes, Inc, OR)
was used. Tris buffer was used in the incubations. In this
5   competitive immunonassay 50 µl standard or sample, was
incubated 100 µl of Compound 2 -labelled antibody (diluted
1/50000) and 100 µl of progesterone-biotin derivative and
tetramethylrodamin-avidin (acceptor). The biotinylated
progesterone competes with progesterone from the standards
10   or the sample for the binding sites on the labelled
anti-progesterone antibody. The reaction mixture was
incubated for 1 hour at room temperature. After the
incubation the fluorescence signal was measured from
tetramethylrodamin-avidin bound to the
15   anti-progesterone-antibody-progesterone-biotin complexes
with a time-resolved fluorometer (Wallac 1420 VICTOR,
excitation at 340 nm, emission at 572 nm, +- 3 nm, delay
time 50 µs, window time 100 µs, cycle time 1 ms). The
measured signal is inversely related to the progesterone
20   concentration in the sample.

Example 8

Homogenous energy transfer receptor assay of IL-2

Purified carrier-free IL-2 (R&D Systems, Minneapolis, MN)
(1 mg/ml) was incubated with a 10-fold molar excess of
25   Compound 2 in 50mM carbonate buffer pH 9.2 over night at +
4 °C. The labelled IL-2 was separated from unreacted
chelate by gel filtration (Sephadex G50, 0.7x48 cm,
Pharmacia) with Tris as elution buffer. The labelled IL-2
was analyzed and found to contain 1.4 molecules of Compound
30   2 per IL-2 molecule. A non-neutralizing antibody directed
against the IL-2α receptor was labelled with
tetramethylrhodamine isothiocyanate according to Example 2.
In the homogenous receptor assay increasing concentrations
of unlabelled IL-2 was co-incubated with constant
35   quantities of Compound 2 -labelled IL-2 (donor), IL-2α

receptor and the tetramethylrhodamine labelled antibody
(acceptor). Tris buffer was used in this incubation. A
displacement curve was constructed by measuring the
fluorescence signal from the formed antibody-receptor-IL-2
5   complexes in a time-resolved fluorometer (Wallac 1420
VICTOR, excitation at 340 nm, emission at 572 nm +- 3 nm,
delay time 50 µs, window time 100 µs, cycle time 1 ms).

Example 9

Double labelling of casein with a Compound 6 and
10   teramethylrhodamine

Five milligrams of casein was dissolved in 0.5 mL of 0.1 M
sodium carbonate pH 9.2. Fifty microliters of 5.5 mM
fluorescent Compound 6 in water was added to the casein
solution and reaction was allowed to proceed overnight at 4
15   °C. The labelled protein was purified through Sephadex G-50
column using 0.9 % sodium chloride in aqueous solution as
an eluent. The labelled casein was analyzed and found to
contain one Compound 6 molecule per casein molecule. Two
milligrams of Compound 6 -labelled casein in 1 mL was mixed
20   with 100 µl of 20 mM tetramethylrhodamine isothiocyanate
(TRITC) dissolved in dimethylformamide. After adjusting pH
by adding 120 µl of 1 M sodium carbonate, pH 9.5, reaction
mixture was incubated at 4 °C overnight. The labelled
protein was purified from unreacted TRITC using Sephadex
25   G-50 column equilibrated and run with 50 mM Tris-HCl pH 8
containing 0.9% NaCl. Using the same protocol as for
Compound 6 -labelled casein, unlabelled casein was also
labelled with TRITC.

Example 10

30   Labelling of Compound 6 -labelled casein with a spin label.

Two milligrams of Compound 6 -labelled casein (Example 9)
in 1 mL 0.9% NaCl was mixed with 110 µl of 1 M sodium

carbonate pH 9.5 and 13 µl of 160 mM
3-(2-(2-Isothiocyanatoethoxy)-ethylcarbamoyl)-PROXYL
(Aldrich) was added. After overnight reaction at 4 °C the
double labelled protein was purified using Sephadex G-50
5   column.

Example 11

Fluorometric properties of double-labelled caseins

The double-labelled proteins from Examples 9 and 10
together with Compound 6 casein (Example 9) were analyzed
10   in a multilabel reader (VICTOR, Wallac Oy). Labelled
caseins were diluted in 50 mM Tris-HCl pH 7.8 to a
concentration of 0.2 µg/mL. Using the protocol for terbium
measurement (excitation 340 nm, emission 545 nm, delay time
0.5 ms, window time 1.4 ms, cycle time 2 ms) Compound 6-
15   labelled casein gave 743 000 counts per second (cps).
Casein labelled with Compound 6 and TRITC gave 28 000 cps,
casein labelled with Compound 6 and PROXYL 54 000 cps and
casein labelled with TRITC 869 cps. When the abovementioned
proteins were measured using excitation at 340 nm, emission
20   at 572 nm +- 3nm (suitable for TRITC), delay time 50 µs,
window time 100 µs and cycle time 1 ms, Compound 6
-labelled casein gave 3952 cps, Compound 6- and
TRITC-labelled casein 382 000 cps, Compound 6- and
PROXYL-labelled casein 4097 cps and TRITC-labelled casein
25   747 cps. These results show that labelling of Compound 6-
labelled casein with TRITC or PROXYL efficiently quenched
Compound 6 -fluorescence. In the case of Compound 6- and
TRITC-labelled casein quenching of Compound 6 -fluorescence
is due to energy transfer between Compound 6 -chelate and
30   rhodamine which can be seen in fluorescence signal using an
emission filter suitable for rhodamine and time-resolved
mode.

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Example 12

Protease assay using double-labelled caseins

Compound 6 -labelled casein, Compound 6- and TRITC-labelled
casein and Compound 6- and PROXYL-labelled casein were
5   diluted in 10 mM Tris-HCl pH 7.6 to a concentration of 50
ng/mL. 180 µl of each diluted protein was pipetted in clear
microtitration wells (NUNC Maxisorp). Fluorescence was
measured using the protocol for terbium measurement.
Following results were obtained: Compound 6 -casein 179 000
10   cps, Compound 6 -TRITC-casein 7860 cps and Compound 6
-PROXYL-casein 14680 cps. Then 100 ng of trypsin in 20 µl
of 10 mM Tris-HCl pH 7.6 was added to each well. After
incubating for 1 hour at room temperature Compound 6
-fluorescence was measured: Compound 6 -casein 163 000 cps,
15   Compound 6 -TRITC-casein 137 000 cps and Compound 6 -PROXYL
-casein 67400 cps. In case of double-labelled caseins there
was up to 17-fold increase in Compound 6 -fluorescence upon
degradation with a protease.

Example 13

20   Homogeneous energy transfer assay for measurement of a
protein kinase activity

Antibody against phosphorylated tyrosine (clone PY20) was
labelled with Compound 4. Incorporation of 3 chelates per
antibody molecule was obtained. Rhodamine-avidin (Rh-Av)
25   and phycoerythrin-avidin (PE-Av) were purchased from Vector
Laboratories. As a substrate we used a peptide with a
following amino acid sequence:
Biotin-Glu-Ala-Ile-Tyr-Ala-Ala-Pro-Phe-Ala-Lys-Lys-Lys. The
biotin moiety was coupled to the amino terminus of the
30   peptide. This peptide is a substrate for Abl protein
tyrosine kinase purchased from New England Biolabs. The
assay was started by incubating 100 nM peptide solution
with labelled avidin (concentration 10 µg/mL). Twenty

microliters of peptide-avidin solution was pipetted to
clear microtitration wells. Next 75 µL of Abl protein
tyrosine kinase assay buffer (New England Biolabs; 50 mM
Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM Na_2EDTA, 1 mM

5   dithiothreitol, 0.015% Brij 35) containing 25 µM ATP and
250 µg/mL Compound 3 -labelled anti-phosphotyrosine was
added to each well. Then either 5 µL of kinase assay buffer
(blank) or 5 µL of Abl protein tyrosine kinase (1 unit)
diluted in assay buffer was added. Measurement was

10   performed in VICTOR reader using time-resolved mode
(excitation at 340 nm, emission at 572 nm +- 3 nm, delay
time 50 µs, window time 100 µs, cycle time 1 ms) to measure
signal from rhodamine or phycoerythrin excited by energy
transfer from Compound 4. After 30 minutes following

15   results were obtained:

Labelled avidin	Blank	Signal with enzyme
Rhodamine-avidin	2649 cps	702 000 cps
Phycoerythrin-avidin	2460 cps	974 000 cps

These results show that kinase activity can be detected

20   using the described assay format based on energy transfer
between Compound 4 and rhodamine or phycoerythrin.

Example 14

Homogeneous energy transfer DNA hybridization assay

The following oligonucleotides were synthesized using

25   Applied Biosystems DNA/RNA synthesizer:
Target oligonucleotide 5'-ACG GTT CTA GCG TTG CAC TAC GGC
TGC AAC TAG CAG TCC-3'
Compound 6 -labelled oligonucleotide 5'-Compound 3-X-GTG
CAA CGC TAG AAC-3'

30   Tetramethylrhodamine-labelled oligonucleotide 5'-TGC TAG
TTG CAG CCG-X-TRITC-3'
Compound 6- and TRITC-labelled oligonucleotides were

synthesized and labelled using the chemistry and protocol
published earlier (P. Dahlen et al. (1994) Bioconjugate
Chem. 5, 268-272). X denotes a modified deoxycytidine
residue carrying an aliphatic primary amino group suitable
5   for labelling with reagents containing, for example,
isothiocyanate group. Compound 6 used in this example is
fluorescent. The labelled probes are able to hybridize with
the target oligonucleotide so that upon hybridization the
fluorescent Compound 6 and tetramethylrhodamine are
10 separated only by two nucleotides in the target
oligonucleotide. In the assays following hybridization
solution was used: 0.6 M sodium chloride, 50 mM Tris-HCl pH
8, 0.1 mM EDTA, 0.1% sodium dodecylsulphate. The probes
were diluted in hybridization solution to give the final
15 concentration of 10 nM each. The target oligonucleotide was
diluted also in hybridization buffer to give the following
concentrations: 1 nM (in the assay 0.1 nM), 5 nM (in the
assay 0.5 nM) and 10 nM (in the assay 1 nM). Hybridization
solution alone was used as a target for blank. Twenty
20 microliters of target oligonucleotide dilutions and blank
was pipetted to clear microtitration wells. The assay was
performed using two replicates. Then 180 µl of
hybridization solution containing the labelled probes was
added to each well. Then the plate was covered with
25 adhesive tape and placed in an incubator set to 37 °C.
After incubation for 30 minutes the plate was measured
using the time-resolved mode (excitation at 340 nm,
emission at 572 nm +- 3 nm, delay time 50 µs, window time
100 µs, cycle time 1 ms). Following results were obtained:

30 Blank   2331 cps
    0.1 nM target   7170 cps
    0.5 nM target   26980 cps
    1 nM target   46300 cps

These results show that this homogeneous assay works well
35 and the energy transfer between the Compound 6 and
rhodamine is efficient when the two probes hybridize to the

36

same target molecule.

Example 15

Energy transfer assay using beads containing acceptor
molecules

5   Anti-βhCG antibody (code M15294) was labelled with Compound
    6 as described in Example 1. Fifty microliter of orange
    fluorescent beads (1.8×10^6 beads/ml) (FluoroSpheres[R], F-8857
    from Molecular Probes Inc.) were incubated with 50 μl
    Compound 6 -labelled antibody (60 μg/ml) in Tris buffer (50
10 mM Tris-HCl, pH 7.4 containing 0.9% NaCl) for 2 hours. In
    the reaction mixture Compound 6 -labelled antibody is
    passively absorbed to the surface of the bead. The
    background was determined by incubating 50 μl of beads with
    50 μl buffer and 50 μl of antibody solution with 50 μl of
15 buffer. The fluorescence from the acceptor molecules in the
    beads excited by energy transfered from Compound 6 was
    measured in the Wallac 1240 VICTOR time-resolved
    fluorometer. Fluorometer setting used were: excitation at
    340 nm, emission at 572 nm +- 3nm, delay time 50 μs, window
20 time 100 μs, cycle time 1 ms. Results:

	Fluorescence 1000xcps
50 μl beads + 50 μl Compound 6 -labelled antibody	5543
50 μl beads + 50 μl buffer	161
25 50 μl Compound 6 -labelled antibody + 50 μl buffer	158

Example 16

Energy transfer assay using beads containing acceptor
molecules

Anti-βhCG antibody (code M15294) was labelled with Compound
30 9 as described in Example 1. Fifty microliter of crimson
    fluorescent beads (1.8×10^6 beads/ml) (FluoroSpheres[R]),

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F-8855 from Molecular Probes Inc.) were incubated with 50
μl Compound 9 -labelled antibody (60 μg/ml) in Tris buffer
(50 mM Tris-HCl, pH 7.4 containing 0.9% NaCl) for 2 hours.
In the reaction mixture Compound 9 -labelled antibody is
5 passively absorbed to the surface of the bead. The
background was determined by incubating 50 μl of beads with
50 μl buffer and 50 μl of antibody solution with 50 μl of
buffer. The fluorescence from the acceptor molecules in the
beads excited by energy transfered from Compound 9 was
10 measured in the Wallac 1240 VICTOR time-resolved
fluorometer. Fluorometer setting used were: excitation at
340 nm, emission at 660 nm +- 1 nm, delay time 100 μs,
window time 100 μs, cycle time 1 ms. Results:

                                                    Fluorescence
15   1000xcps

50 μl beads + 50 μl Compound 9 -labelled antibody 1398
50 μl beads + 50 μl buffer   8
50 μl Compound 9 -labelled antibody + 50 μl buffer 102

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It will be appreciated that the methods of the present
invention can be incorporated in the form of a variety of
embodiments, only a few of which are disclosed herein. It
will be apparent to the artisan that other embodiments
25 exist and do not depart from the spirit of the invention.
Thus, the described embodiments are illustrative and should
not be construed as restrictive.

CLAIMS

1. A luminescence energy transfer assay comprising a first
group labelled with an energy donor and a second group
labelled with an energy acceptor, wherein
- the donor is a long excited state lifetime luminescent
5   lanthanide chelate and the acceptor is either a short
excited state lifetime luminescent label or a non-
luminescent label, and
- and the increase or decrease, respectively, in energy
transfer from the donor label to the acceptor label
10   resulting from shortening or lengthening, respectively, of
the distance between said labels, is measured
characterized in that the long excited state lifetime
luminescent lanthanide chelate has one or more of the
following properties:
15   - a high luminescence yield,
- an excited state lifetime of 1 ms or more, and
- an emission distribution optimized for energy transfer.

2. A luminescence energy transfer assay comprising a first
group labelled with an energy donor and a second group
20   labelled with an energy acceptor, wherein
- the acceptor is either a short excited state lifetime
luminescent label or a non-luminescent label, and
- and the decrease in energy transfer from the donor label
to the acceptor label resulting from lengthening of the
25   distance between said labels, is measured
characterized in that the donor is a long excited state
lifetime luminescent lanthanide chelate.

3. A luminescence energy transfer assay comprising a first
group labelled with an energy donor and a second group
30   labelled with an energy acceptor, wherein
- the acceptor is either a short excited state lifetime
luminescent label or a non-luminescent label, and
- and the increase or decrease, respectively, in energy

transfer from the donor label to the acceptor label
resulting from shortening or lengthening, respectively, of
the distance between said labels, is measured
characterized in that the donor is a luminescent lanthanide
5 chelate, and
- two or more analytes are simultaneously measured, wherein
each analyte is measured by a specific donor-acceptor pair
and the donor and acceptor are selected from those defined
above.

10 4. A luminescence energy transfer assay comprising a first
group labelled with an energy donor and a second group
labelled with an energy acceptor, wherein
- the acceptor is either a short excited state lifetime
luminescent label or a non-luminescent label, and
15 - and the increase or decrease, respectively, in energy
transfer from the donor label to the acceptor label
resulting from shortening or lengthening, respectively, of
the distance between said labels, is measured
characterized in that the donor is a luminescent lanthanide
20 chelate, and
- the luminescent or non-luminescent label of the acceptor
is attached to a solid carrier providing a large surface to
which said second group is bound or can be bound via
suitable covalent or non-covalent means.

25 5. The assay according to claim 4 _characterized_ in that the
luminescent or non-luminescent label molecules are in the
solid carrier.

6. The assay according to claim 4 _characterized_ in that the
solid carrier is surface activated microbeads covalently
30 coupled with the second group and with a plurality of
acceptor molecules.

7. The assay according to claim 4 _characterized_ in that the
surface has been made by coating the microbeads with an
adherent macromole such as a polylysine, agglutinin,

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lectin, antibody, which macromolecule either prior to or
after the coating step is conjugated with the acceptor
molecules.

8. A luminescence energy transfer assay comprising a first
5   group labelled with an energy donor and a second group
labelled with an energy acceptor, wherein
- the acceptor is either a short excited state lifetime
luminescent label or a non-luminescent label, and
- and the increase or decrease, respectively, in energy
10 transfer from the donor label to the acceptor label
resulting from shortening or lengthening, respectively, of
the distance between said labels, is measured
characterized in that the donor is a luminescent lanthanide
chelate, and
15 - the acceptor is lipophilic compound anchored to a cell
membrane or cell membrane fragment or the acceptor is made
lipophilic by conjugating with a long chain hydrocarbon
such as a fatty acid.

9. The assay according to any of the claims 2 - 8
20 characterized in that the luminescent lanthanide chelate
has a long excited state lifetime and that it has one or
more of the following properties:
- a high luminescence yield,
- an excited state lifetime of 1 ms or more, and
25 - an emission distribution optimized for energy transfer.

10. The assay according to any of the claims 1 and 3 - 9
characterized in that the assay is based on a bioaffinity
binding reaction.

30 11. The assay according to any of the claims 1 - 10
characterized in that the assay is based on an
enzymatically catalyzed reaction.

12. The assay according to claim 11 characterized in that
the reaction is a cleavage reaction.

13. The assay according to any of the foregoing claims
<u>characterized</u> in that the lanthanide of the donor chelate
is europium, terbium or samarium.

14. The assay according to claim 13 <u>characterized</u> in that
5 the energy transfer is registered by a time-resolved
fluorometer using a predeterminated instrumental setting
avoiding cross talk or donor emission to the channel used
for acceptor emission measurement when there is no energy
transfer.

10 15. The assay according to claim 14 <u>characterized</u> in that
the instrumental counting time window is optimized for the
assay, having delay period long compared to the length of
excitation pulses and the emission lifetime of the acceptor
without energy transfer, and the counting window shorter
15 than the decay of the donor in assays measuring decreased
distances.

16. The assay according to any of the foregoing claims
<u>characterized</u> in that the acceptor molecules are brought to
the vicinity of specific binding reaction using suitable
20 secondary affinity reactions, such as anti-receptor
antibodies, use of biotinylated binding reagents and
acceptor labeled avidin, lectins etc.

17. The assay according to any of the claims 1 - 9 or 11 -
16 <u>characterized</u> in that the acceptor is a spin label.

25 18. A luminescence energy transfer assay comprising a first
group labelled with an energy donor and a second group
labelled with an energy acceptor, wherein
- the acceptor is either a short excited state lifetime
luminescent label or a non-luminescent label, and
30 - and the increase or decrease, respectively, in energy
transfer from the donor label to the acceptor label
resulting from shortening or lengthening, respectively, of
the distance between said labels, is measured

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<u>characterized</u> in that the donor is a luminescent lanthanide chelate, and that the instrument used to measure energy transfer is able to follow multiple parameters simultaneously or consecutely, such as absorbances at the

5 wavelenghts of excitation or emissions, luminescence of different durations, scattering and luminescence decay times, in order to monitor and correct any interferences the sample matrix may have to excitation, energy transfer or emissions.

10 19. The assay according to claim 18 <u>characterized</u> in that the instrument automatically corrects any attenuation of excitation the sample may cause by following simultaneously the absorbances of the samples diluted into assay mixture and correcting the emission readings according to

15 excitation or emission attenuation by sample absorption.

20. The assay according to claim 18 <u>characterized</u> in that the measured acceptor signal is normalized by recording also the donor emission which can be compared to zero calibrator, the lowering of signal without concomitant

20 increase in acceptor luminescence being recorded and corrected.

21. The assay according to claim 18 <u>characterized</u> in that the decay time of either donor or the acceptor is registered and used as the measuring parameter.

25 22. The assay according to claim 18 <u>characterized</u> in that the decay time of the donor or the acceptor is utilized in correction of sample interferences.

23. The assay according to claim 18 <u>characterized</u> in that it is a suitable combination of the measurements according
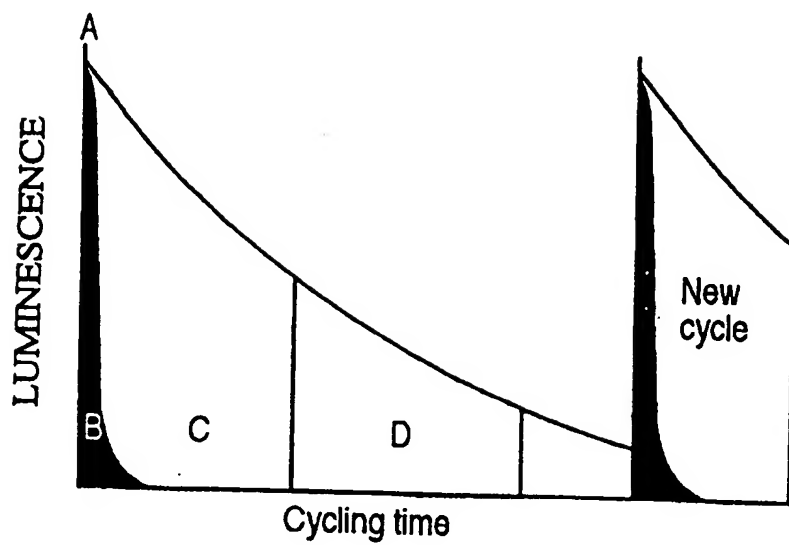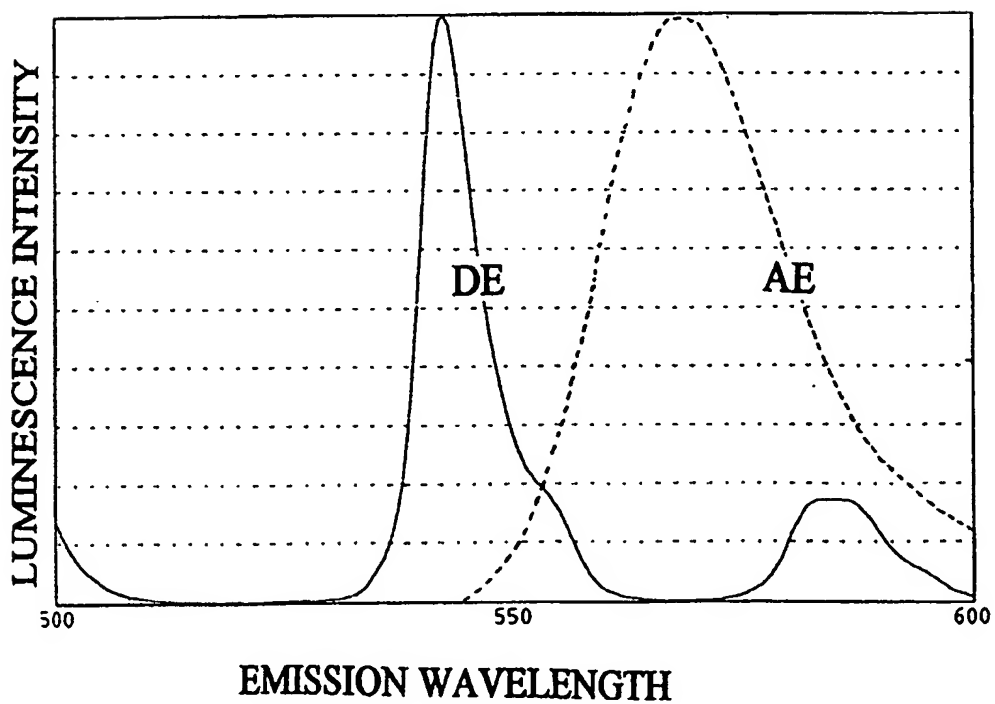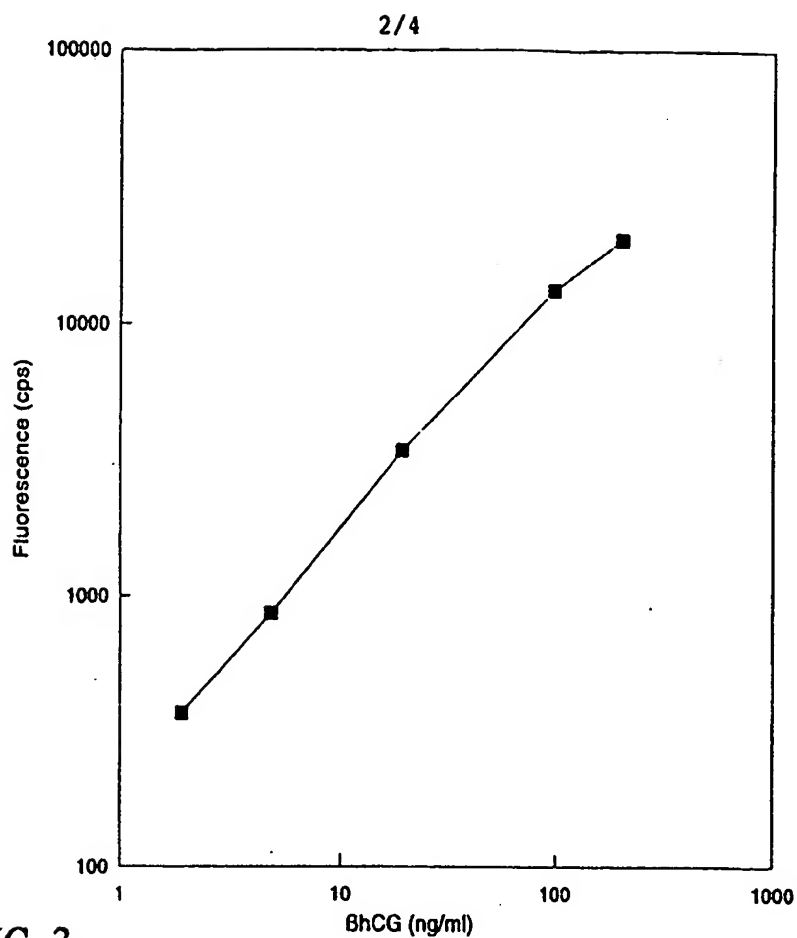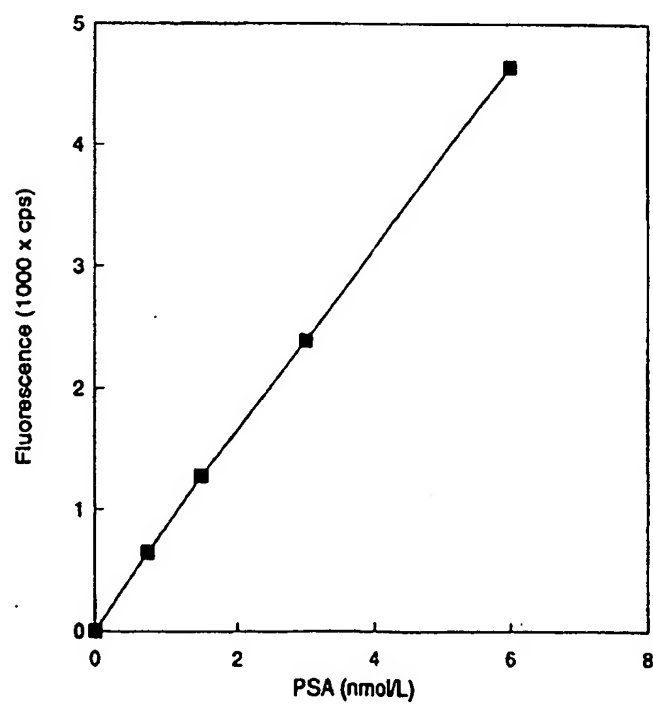
30 to claims 19, 20, 21 or 22.

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

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FIG. 6